

Synthetic hydrogel niches that promote hMSC viability

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Abstract

Photopolymerized poly(ethylene glycol) (PEG) hydrogels were used as a base platform for the encapsulation and culture of human mesenchymal stem cells (hMSCs). The base PEG formulation presents an environment completely devoid of cell–matrix interactions. As such, viability of hMSCs in unmodified PEG hydrogels is very low. This formulation was modified to contain pendant phosphate groups to facilitate the sequestering of osteopontin within the gel, as well as pendant cell-adhesive RGD peptide sequences, which are found in osteopontin and other cell adhesion proteins. The survivability of hMSCs was examined with culture time and as a function of the gel chemistry to examine the role of cell–matrix interactions in promoting long-term viability. In the absence of any adhesive ligands, hMSC viability drops to 15% after 1 week in culture. However, by incorporating the RGD sequence or pendant phosphate groups this low viability was rescued to 75% and 97%, respectively. It is believed that the phosphate groups promote mineralization of the hydrogel network, and this mineral phase sequesters cell-secreted osteopontin, resulting in enhanced cell–matrix interactions and improved cell viability. Published by Elsevier B.V./International Society of Matrix Biology.

Keywords: Hydrogel; Human mesenchymal stem cells; Photoencapsulation; Osteopontin; Cell viability; Poly(ethylene glycol)

1. Introduction

Hydrogels are water swollen, but insoluble, polymer networks. The high water content and tissue-like elasticity lead to properties that are similar to many tissues, and as such, hydrogels are often explored as synthetic extracellular matrix analogs for the three-dimensional culture of cells. Hydrogels can be synthesized from purely synthetic components that create an environment that limits cellular interactions and only cell secreted extracellular matrix molecules can modify this niche. In contrast, chemical derivatization of hydrogels (e.g., with peptide epitopes or adhesion proteins) affords opportunities to control cell–matrix interactions in a highly controlled manner. In essence, these two situations allow the creation of cellular

niches that simply permit cells to function or actively present cues that promote selected functions. To date, researchers have explored the development of hydrogels for the delivery of numerous primary cells, including chondrocytes (Bryant and Anseth, 2001, 2002; Bryant et al., 2004; Paige et al., 1995; Rice and Anseth, 2004; Sittering et al., 2004), osteoblasts (Burdick and Anseth, 2002; Burdick et al., 2002; Sittering et al., 2004), valvular interstitial cells (Masters et al., 2004), smooth muscle cells (Mann, 2003; Mann et al., 2001; Moffat and Marra, 2004; Ramamurthi and Vesely, 2005), fibroblasts (De Rosa et al., 2004; Shu et al., 2004; Zielinski and Aebischer, 1994), and mesenchymal stem cells (Lawson et al., 2004; Nuttelman et al., 2004; Schantz et al., 2003; Temenoff et al., 2004; Wang et al., 2003; Williams et al., 2003).

An emerging area of interest is the design of hydrogel carriers for therapeutic applications, such as the three-dimensional culture and expansion of stem cells, particularly mesenchymal stem cells (MSCs). For regenerative medicine applications, MSCs have many

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advantages including their ease of isolation (Ballas et al., 2002), high proliferative capacity (Banfi et al., 2002; Bruder et al., 1997a,b; Haynesworth et al., 1992a,b), and ability to differentiate into a variety of cell types that are capable of producing large amounts of tissue. Under the right environmental stimuli, MSCs are capable of differentiating into the cells of bone, cartilage, tendon, muscle, fat, and others (Bruder et al., 1997a,b; Caplan, 1991; Haynesworth et al., 1992a,b; Pittenger et al., 1999). Because of these properties, numerous groups are exploring the development of scaffolds for three-dimensional MSC culture. Interestingly, when MSCs are encapsulated in a permissive hydrogel environment (i.e., one that provides no cues, but simply permits cells to function), such as poly(ethylene glycol) (PEG), their viability drops off dramatically with culture time (Nuttelman et al., 2004). Specifically, less than 10% of human mesenchymal stem cells (hMSCs) survive after 4 weeks of culture in PEG hydrogels. This observation is in stark contrast to previous cell encapsulation studies with bovine chondrocytes (Bryant and Anseth, 2001), where cell viability remained high (>95%) after 6 weeks in culture, and neonatal rat calvarial osteoblasts (Burdick and Anseth, 2002), where cell viability was greater than 80% after 4 weeks in culture, in similar PEG hydrogels. Part of this difference may be related to the differences in anchorage dependence, species, and age of the various cells; however, in regards to hMSCs, interesting results have been reported with respect to expression of cell adhesive protein genes, especially osteopontin, when hMSCs are cultured in permissive PEG hydrogels.

Osteopontin is an especially important cell adhesion protein for hMSCs, regulating both adhesion of osteoprogenitor cells and the differentiation of stem cells to osteoblasts (Darimont et al., 2002; Ohgushi and Caplan, 1999; Yabe et al., 1997). Adhesion of osteoprogenitor cells to the bone surface during remodeling is one of the major turning points in their differentiation to mature osteoblasts; during this differentiation process, osteoprogenitor cells have been reported to make large amounts of bone tissue quickly (Yabe et al., 1997). When hMSCs were encapsulated in PEG hydrogels and cultured in basal media, osteopontin expression by these cells after 1 week was found to be nearly ten times higher than osteopontin expression by hMSCs cultured in monolayer culture (Nuttelman et al., 2004). In general, cells do not attach directly to highly hydrated synthetic gels, such as PEG, because of limited protein adsorption, so hMSCs encapsulated inside these gels are presented with a “blank” environment. We hypothesize that the encapsulated hMSCs are secreting elevated levels of osteopontin in an effort to modify their environment to enable cell attachment.

While cells can normally alter their environment by secreting adhesion proteins, there is little to no inter-

action of secreted adhesion proteins with the surrounding environment once the cells are encapsulated within a PEG gel and osteopontin can readily diffuse through the gel. As a result, there are few interactions between cell–surface integrins and the surrounding gel environment. Cell–matrix interactions can dramatically affect differentiation, survival, and tissue evolution; anchorage-dependent osteoprogenitor cells, such as hMSCs require a support matrix in order to survive (Ishaug-Riley et al., 1998). In the absence of cell–matrix interactions, anchorage-dependent cells undergo apoptosis, or more specifically, anoikis [i.e., apoptosis that occurs as a result of cell detachment from the extracellular matrix (Frish and Ruoslahti, 1997)]. Cell binding to the extracellular matrix through integrin receptors is thought to provide signals for suppression of apoptosis (Ruoslahti and Reed, 1994). Thus, when designing hydrogel niches to serve as synthetic extracellular matrix environments, preservation of matrix–integrin interactions is critical to promote long-term cell survival and function. In fact, nonspecific cell attachment to a substrate is not sufficient to prevent apoptosis; specific binding to integrins is required to prevent apoptosis. This specificity has been demonstrated using surface-immobilized antibodies against two types of non-integrin cell surface molecules, which did not prevent apoptosis, as opposed to surface-immobilized anti-integrin antibodies, which did lead to prevention of apoptosis (Meredith et al., 1993).

In this research, we aimed to better understand the role of the extracellular matrix in supporting hMSC viability and survivability by designing two specific types of hydrogel niches that incorporate functionalities designed to enhance specific matrix–integrin interactions. The first gels contain a pendant arginine–glycine–aspartic acid (RGD) peptide sequence that is found in numerous cell adhesion proteins, including osteopontin (Ruoslahti, 1996). The RGD sequence is known to bind to integrin receptors on cell surfaces, supporting cell adhesion and preventing apoptosis (Frish and Ruoslahti, 1997; Ruoslahti, 1996; Ruoslahti and Reed, 1994; Wadsworth et al., 2004). The second gels were designed to capture and sequester cell-secreted osteopontin. Osteopontin, like other sialoproteins, has an acidic domain that binds to the positively charged hydroxyapatite mineral matrix of bone. Cells attach to bone mainly through osteopontin that, through electrostatic interactions, has adsorbed to the mineralized matrix. Thus, negatively charged phosphate groups were incorporated into the PEG gel to indirectly promote cell adhesion by facilitating mineralization and subsequent adsorption of cell-secreted osteopontin. Through these two hydrogel formulations, we aimed to understand the role of matrix adhesion, as well as the relative ability of a peptide sequence versus an entire protein, to promote long-term survivability, and presumably prevent apoptosis, of encapsulated hMSCs.

2. Results and discussion

2.1. PEG–RGD hydrogels: effect on two-dimensional hMSC attachment

The RGD (arginine–glycine–aspartic acid) sequence is a highly conserved sequence that is found in many adhesion proteins, including fibronectin, laminin, and osteopontin. Integrin receptors found on the cell membrane specifically recognize the RGD sequence, allowing cells to bind to their extracellular matrix environment. Moreover, the binding of integrins to cell adhesion proteins and the RGD sequence leads to a conformational change of the integrin receptors, ultimately leading to a signaling cascade that results in the prevention of apoptosis (Meredith et al., 1993; Ruoslahti and Reed, 1994). Other researchers have tethered the RGD sequence to a photopolymerizable, acrylated PEG molecule (PEGDA) to promote cell attachment to photopolymerizable PEG-based or oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels (Benoit and Anseth, 2005; Burdick and Anseth, 2002; Hern and Hubbell, 1998; Mann et al., 2001; Shin et al., 2004a,b; VandeVondele et al., 2003). In the experiments presented here, it was hypothesized that, by tethering the RGD sequence to a two-dimensional PEGDA surface, hMSC attachment to and spreading on PEGDA surfaces would be improved due to the formation of integrin-tethered RGD interactions. Fig. 1 shows hMSCs that have been seeded on PEGDA hydrogels containing no Acryl–PEG–RGD (top) and 2.8 mM Acryl–PEG–RGD (bottom). Cell attachment and spreading were improved dramatically when cells were seeded on RGD-modified PEGDA hydrogels. Moreover, the cells maintain their phenotype and are viable and proliferate on the hydrogels for extended periods of time, indicating that RGD-modified gels are suitable for the long-term culture of hMSCs.

Considering this result, viability of hMSCs photoencapsulated in PEGDA hydrogels containing Acryl–PEG–RGD was investigated and compared to unmodified PEGDA hydrogels.

2.2. PEG–RGD hydrogels: effect on three-dimensional hMSC culture

Human MSCs were photoencapsulated in PEGDA hydrogels in the presence of 2.8 mM Acryl–PEG–RGD. During photoencapsulation, this molecule is reacted into the network through a free radical reaction of the methacrylate carbon–carbon double bond. The hypothesis is that hMSCs that have been encapsulated in the PEGDA hydrogel in the presence of the tethered RGD peptide will interact with the RGD sequence at the cell/hydrogel interface and this interaction will promote hMSC survival. Integrin receptors on the cell membrane can bind to the tethered RGD peptides, mimicking integrin/matrix interactions and suppressing apoptosis. Fig. 2 shows the differences between hMSCs photoencapsulated in unmodified PEGDA hydrogels (left) and in PEGDA hydrogels modified with 2.8 mM Acryl–PEG–RGD (right). Cell viability of hMSCs improved from around 15% when encapsulated in the unmodified PEGDA hydrogel to around 75% in RGD-modified PEGDA scaffolds after 1 week in culture.

2.3. Osteopontin adsorption to pre-mineralized PEG hydrogels

Although the RGD sequence is a very important region of most cell adhesion proteins, it is believed that optimal cell adhesion results through synergy of various peptide regions on cell adhesion proteins. Considering this, there are thought to be advantages to having the entire cell adhesion

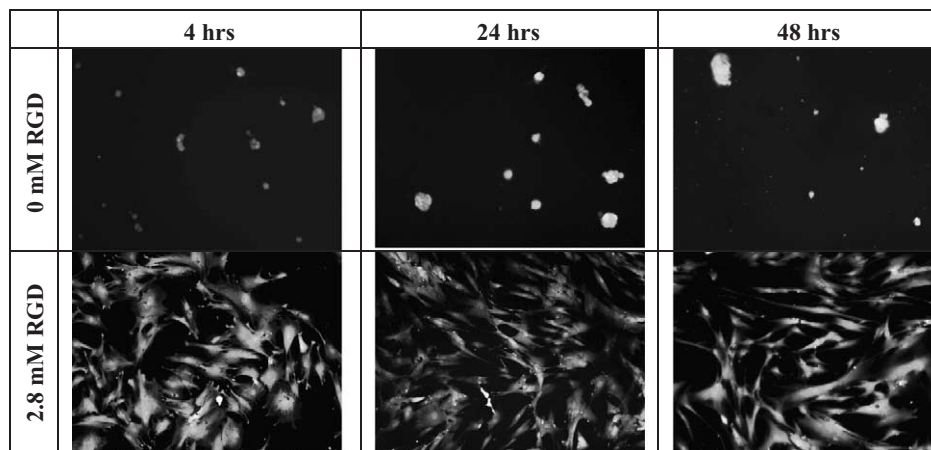


Fig. 1. Human MSCs were seeded onto various two-dimensional PEG surfaces at a concentration of 5000 cells/cm². At various time points, cells were stained with calcein AM in order to visualize the attachment and spreading of cells on the substrates. Shown are cells that have been seeded up to 48 h on PEG3400DA only (top) and PEG3400DA with 2.8 mM Acryl–PEG3400–RGD (bottom) (shown in grayscale, 100X original magnification). Cell attachment and spreading are improved on hydrogels containing RGD.

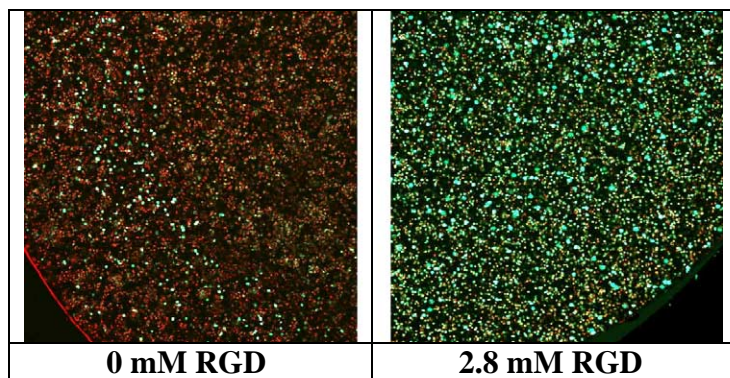


Fig. 2. Effect of RGD incorporation on hMSC viability inside PEGDA hydrogels. Human MSCs were encapsulated in unmodified PEGDA (left) or PEGDA containing 2.8 mM Acryl-PEG-RGD (right) and cultured for 1 week in CON media. Viability was assessed using the LIVE/DEAD assay ($\times 50$ original magnification). Incorporating the RGD peptide sequence improves viability from about 15% in the unmodified gel to around 75% in the RGD-modified gel after 1 week in culture.

protein present to improve cell adhesion. When PEG hydrogels are cultured in osteogenic (OST) differentiation media, the β -glycerophosphate present causes nucleation of mineralized regions throughout the hydrogel. Over time, the gel mineralizes and becomes opaque. It was observed that the viability of encapsulated hMSCs was improved after 1 week when cultured in OST media ($\sim 47\%$ viability) as compared to CON media ($\sim 15\%$ viability), even in the interior of the construct, as shown in Fig. 3. This indicates that mineralization of PEG hydrogels leads to improved cell viability. We hypothesized that osteopontin would adsorb much stronger to mineralized PEGDA hydrogels, leading to improved cell attachment. To test this hypothesis, the ability of osteopontin to adsorb to three different hydrogel formulations was investigated using an ELISA-like assay. Fig. 4 shows the relative osteopontin adsorption to gels with no mineralization (A, $0 \mu\text{g Ca}^{2+}/\text{mg gel}$), moderate mineralization (B, $11 \pm 2 \mu\text{g Ca}^{2+}/\text{mg gel}$), and high mineralization (C, $25 \pm 3 \mu\text{g Ca}^{2+}/\text{mg gel}$). The extent of mineralization was controlled by varying the time that the gels were allowed to mineralize in OST media. As can be

seen, there is much greater osteopontin adsorption to the mineralized hydrogels than the non-mineralized hydrogels. These results indicated that hMSC adhesion would be improved on mineralized hydrogels over non-mineralized hydrogels, since it was believed that osteopontin adsorption to the mineralized gels would lead to an increase in cell adhesion.

2.4. Human MSC attachment to pre-mineralized PEG gels

To determine whether cells would adhere better to the non-mineralized or pre-mineralized surfaces, hMSCs were seeded onto the same gel compositions shown in Fig. 4, and the results are shown in Fig. 5. The top photographs show light microscopy photographs of hMSCs after 4 h of seeding, and the bottom images are confocal images of seeded cells stained using the LIVE/DEAD assay 4 h after cell seeding. As can be seen, cell attachment onto the non-mineralized surfaces is low as compared to cell attachment and spreading on the pre-mineralized surfaces. This result supports our hypothesis that cell adhesion is improved on

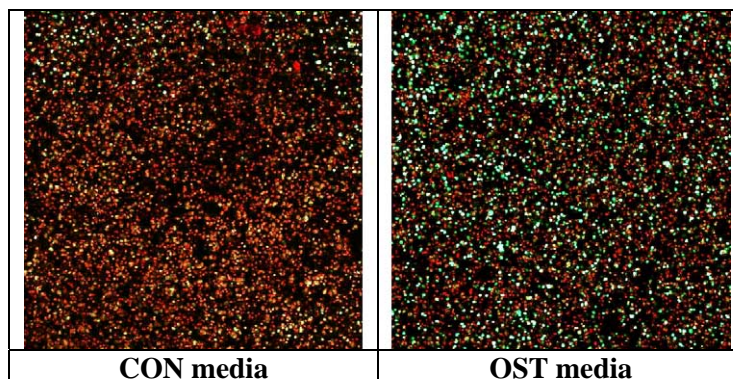


Fig. 3. Effect of media on cell viability. Human MSCs were photoencapsulated in PEG3400DA hydrogels and constructs were cultured in hMSC control (CON) media (left) or osteogenic (OST) differentiation media (right) for 1 week. Viability of encapsulated cells was assessed using the LIVE/DEAD assay. Confocal imaging was utilized to visualize the distribution of living and dead cells in the interior of the gel ($\times 50$ original magnification). In the presence of osteogenic media (which contains mineralization nucleators), cell viability is much improved over constructs cultured in CON media from around 15–47%, indicating that mineralization may enhance cell viability.

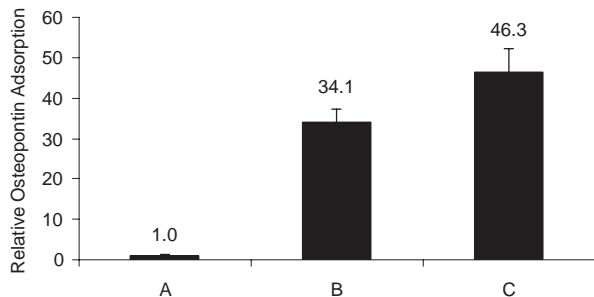


Fig. 4. Relative osteopontin adsorption to pre-mineralized PEGDA hydrogels. PEGDA hydrogels were pre-mineralized by placing in OST media for various time points and were found to have the following calcium content: A) $0 \mu\text{g Ca}^{2+}/\text{mg gel}$; B) $11 \pm 2 \mu\text{g Ca}^{2+}/\text{mg gel}$; C) $25 \pm 3 \mu\text{g Ca}^{2+}/\text{mg gel}$.

mineralized surfaces, and this improvement in cell attachment and spreading on mineralized surfaces is likely due to increased osteopontin adsorption.

2.5. Phosphate-containing gels for osteopontin sequestering

Ultimately, we would like to develop a hydrogel scaffold system for the photoencapsulation of hMSCs that could be formed in situ and actively promote specific developmental pathways and tissue evolution. In the body, it is unlikely that soluble mineralization nucleators, such as β -glycerophosphate, would be present. Therefore, we

developed a novel method whereby phosphate groups are co-polymerized into the network using ethylene glycol methacrylate phosphate (EGMP). During free-radical crosslinking of the network during photopolymerization, EGMP is covalently attached to the evolving hydrogel network through its electron-rich carbon-carbon double bond, resulting in the incorporation of pendant phosphate groups throughout the gel. In the presence of soluble calcium ions, mineralization occurs over time, similar to when β -glycerophosphate is present in the media as a soluble nucleator.

In Fig. 6, 10 wt.% PEGDA hydrogels containing various concentrations of EGMP were synthesized and cultured in hMSC CON media for 2 weeks. Fig. 6 shows qualitatively the differences in mineralization as a function of phosphate content through macroscopic photographs of the various gels. As can be seen, mineralization only occurred when the EGMP concentration was higher than $\sim 5 \text{ mM}$. In addition, mineralization occurred only when serum was present in the media (results not shown).

2.6. Human MSC attachment to phosphate-gel surfaces

The experiments above demonstrated that hMSCs would adhere much better to mineralized surfaces than to non-mineralized surfaces, and these mineralized surfa-

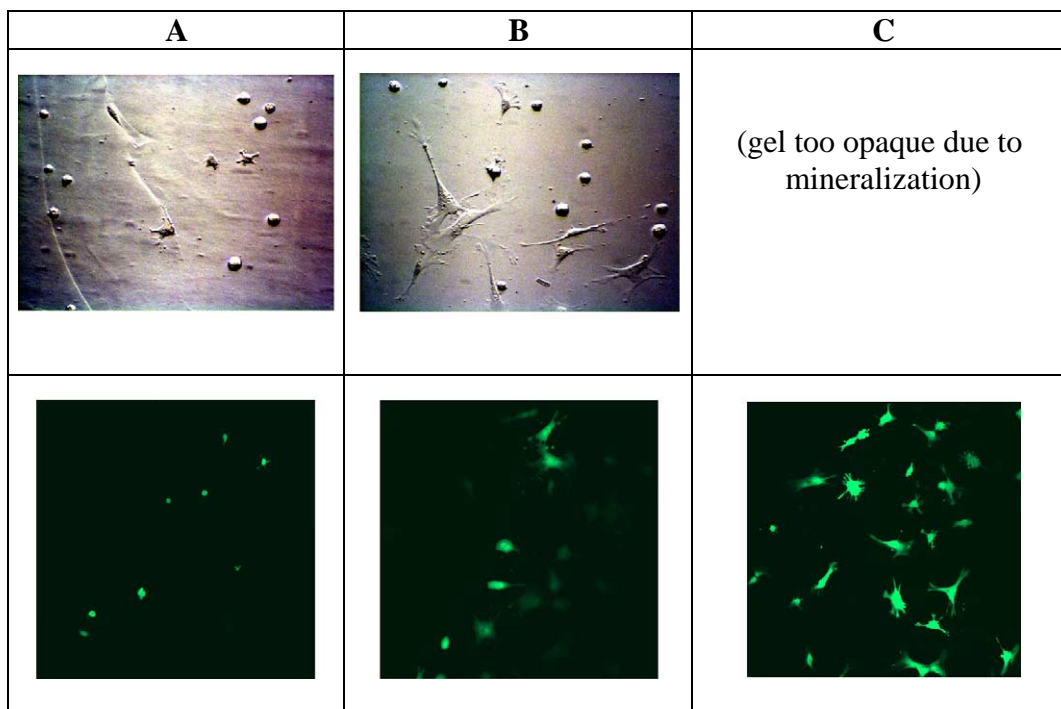


Fig. 5. Human MSCs were seeded on two-dimensional PEGDA hydrogels shown in Fig. 4 at a concentration of $5000 \text{ cells}/\text{cm}^2$. These hydrogels contained $0 \mu\text{g Ca}^{2+}/\text{mg gel}$ (A), $11 \pm 2 \mu\text{g Ca}^{2+}/\text{mg gel}$ (B), and $25 \pm 3 \mu\text{g Ca}^{2+}/\text{mg gel}$ (C). Cells were stained using the LIVE/DEAD assay after 4 h. The ability of hMSCs to attach and spread on the various surfaces was measured using microscopy. Top images are light micrographs ($\times 100$ original magnification) and the bottom images are confocal microscopy images using LIVE/DEAD assay ($\times 200$ original magnification). With increasing mineralization of the PEGDA hydrogels, cell adhesion is improved due to the adsorption of osteopontin. The high mineralization and opaqueness prevented light microscopy from being performed on sample C.

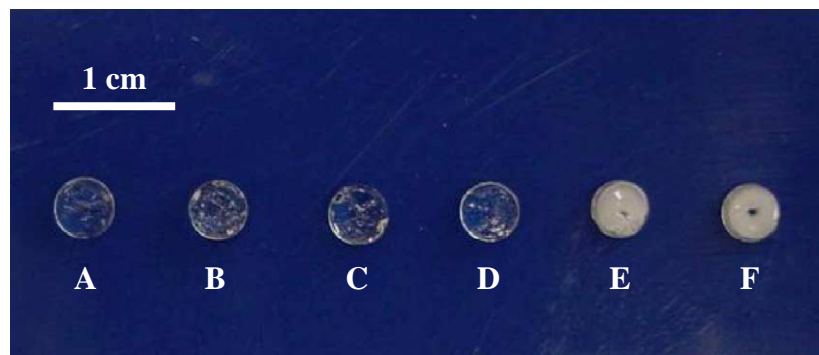


Fig. 6. EGMP was co-polymerized into 10 wt.% PEGDA hydrogels at various concentrations and cultured in media for 2 weeks. Shown is a macroscopic photograph of various EGMP-containing hydrogels after 2 weeks of culture in DMEM media containing 10% fetal bovine serum. A) 0 μ M, B) 10 μ M, C) 100 μ M, D) 1 mM, E) 5 mM, and F) 10 mM EGMP. The only gels with measurable calcium content were E, which contained $242 \pm 25 \mu\text{g Ca}^{2+}/\text{gel}$, and F, which contained $264 \pm 15 \mu\text{g Ca}^{2+}/\text{gel}$.

ces were created by incubating the surfaces in OST media for up to 10 days. However, when cells are encapsulated in PEG hydrogels, there are few mineralization nucleators and the interactions with the gel are almost instantaneous; initially, there will be very little mineralization since it takes time for the gels to mineralize. To speed up mineralization, the negatively charged, photoreactive molecule ethylene glycol methacrylate phosphate (EGMP) was chosen and copolymerized with PEGDA at a concentration of 50 mM to form two-dimensional hydrogel sheets. Human MSCs were seeded onto disks of the EGMP-containing PEGDA hydrogels, and cell morphology was observed after 4, 24, and 48 h. As can be seen in the top of Fig. 7, hMSCs do not adhere to unmodified PEGDA hydrogels, even after 48 h in culture. This lack of adhesion is due to the fact that osteopontin and other cell adhesion proteins do not adsorb strongly to the hydrophilic PEG hydrogel. However, when charge is incorporated into the gels through the use of ethylene

glycol methacrylate phosphate (EGMP, bottom of Fig. 7), there is a dramatic improvement in cell attachment to the PEG hydrogel. It is believed that the negative charges attract positively charged calcium ions from the media. These divalent calcium cations then bind to other negatively charged divalent anions, such as free phosphate groups (PO_4^{2-}), creating calcium-phosphate crystalline regions, much like the hydroxyapatite mineral phase of bone. Over time, gels containing EGMP begin to mineralize, as evidenced by opaqueness and an increase in calcium content. Importantly, serum is also needed in the media for mineralization to occur. We hypothesize that osteopontin secreted by seeded hMSCs adsorbs through electrostatic interactions to these calcium-phosphate, or hydroxyapatite, regions that form, enabling the attachment of hMSCs to EGMP-containing PEG hydrogels. Integrin–matrix interactions form, allowing cells to adhere to the hydrogel. Consequently, this suppresses apoptosis and leads to improved long-term cell viability over unmodified

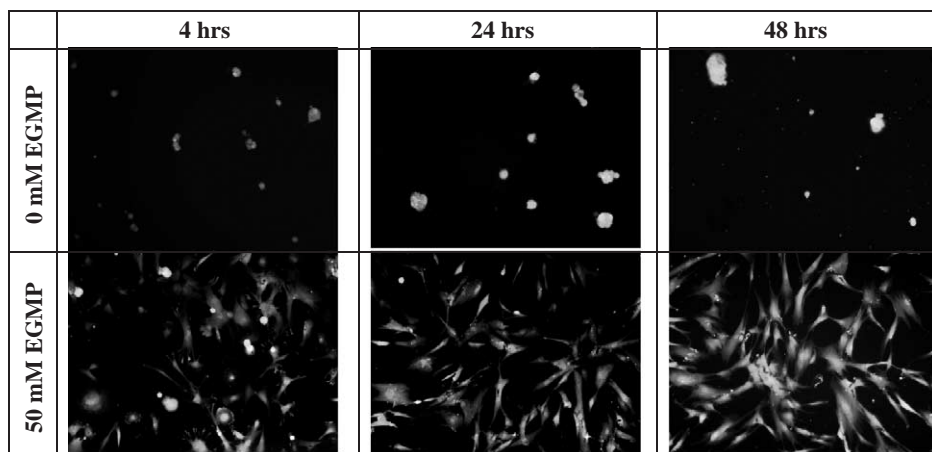


Fig. 7. Human MSCs were seeded onto various two-dimensional PEG surfaces at a concentration of $5000 \text{ cells}/\text{cm}^2$. At various time points, cells were stained with calcein AM in order to visualize the attachment and spreading of cells on the various substrates. Shown are cells that have been seeded for 48 h on PEG3400DA only (top) and PEG3400DA with 50 mM ethylene glycol methacrylate phosphate (EGMP) (bottom) (shown in grayscale, $\times 100$ original magnification). Surfaces were incubated in CON media overnight; if surfaces were incubated in PBS instead, cells would not adhere to the charged surfaces.

PEGDA hydrogels. This hypothesis that divalent calcium ions in solution are contributing strongly towards the attachment of cells to the negatively charged PEG networks is further supported by the fact that if the calcium content in the media is dramatically lowered, then cell attachment is limited (results not shown). Alternatively, if two-dimensional surfaces are incubated in calcium-free PBS instead of calcium-containing media the night prior to cell seeding, cells do not immediately attach to the EGMP surfaces.

2.7. Human MSC viability in osteopontin-sequestering gels

Since incorporating EGMP into PEGDA hydrogel surfaces dramatically improves cell attachment and viability over unmodified PEGDA, the next step was to determine whether viability of three-dimensionally photoencapsulated cells would increase when EGMP was included in the network. As can be seen in Fig. 8, with no EGMP (left), cell viability of encapsulated hMSCs after 1 week is low (15% viability). However, when cells are encapsulated in the presence of 10 mM EGMP, cell viability after 1 week is improved, with around 74% viability (middle). Cell viability increases to 97% (right) after 1 week in culture when 50 mM EGMP is copolymerized with the cells during photoencapsulation. As discussed above, it is believed that the negatively charged phosphate groups recruit positively charged, divalent calcium ions in solution, creating a calcium-phosphate mineral phase, which provides cell attachment sites immediately outside the cell where the cell contacts the PEGDA hydrogel. The ability of integrin receptors on the cell membrane to interact with the secreted, immobilized osteopontin then provides signals that prevent the cell from undergoing apoptosis, improving cell viability.

Table 1 summarizes the cell viability findings. A gel consisting of 50 mM EGMP supports the highest cell viability that was observed, with approximately 97% of the cells remaining alive after 1 week in culture. Furthermore,

Table 1

Summary of viability results

Gel formulation	CON media	OST media	Percentage of viable cells at 1 week (average \pm standard deviation)
Un-modified PEGDA	X		15 \pm 7
Un-modified PEGDA		X	47 \pm 5*
2.5 mM Acryl-RGD	X		75 \pm 13*
10 mM EGMP	X		74 \pm 5*
50 mM EGMP	X		97 \pm 7**

Human MSCs were photoencapsulated in PEGDA hydrogels in the presence of either a tethered RGD peptide sequence (Acryl-RGD) or one of two concentrations of ethylene glycol methacrylate phosphate (EGMP, 10 or 50 mM) and cultured in CON media for 1 week. Viability of cells encapsulated within the gel was assessed using the LIVE/DEAD assay. Three separate photographs were obtained using confocal microscopy, and living and dead cells were counted, averaged, and the error was estimated using the standard deviation of the mean. Additionally, unmodified gels were cultured in OST media for 1 week and analyzed similarly for cell viability. (*Viability significantly higher than unmodified PEGDA cultured in CON media, $p < 0.01$; **Viability significantly higher than all other samples, $p < 0.01$).

high cell viability is observed for at least up to 4 weeks in culture in gels containing 50 mM EGMP (results not shown). While it appears that the 50 mM EGMP gels result in better viability than the 2.8 mM RGD gels, they should not be directly compared. It is entirely likely that an increase in RGD concentration would lead to an even improved viability. On the other hand, it is likely that there are benefits to the entire molecule being present rather than just the RGD sequence; moreover, it is possible that the mineralized regions promoted by the EGMP are able to sequester other important extracellular matrix proteins, such as growth factors and collagen, leading to further improvement in cell viability. Histological staining indicates that osteopontin is located in pericellular regions immediately outside the cell when EGMP is included in the gel (results not shown).

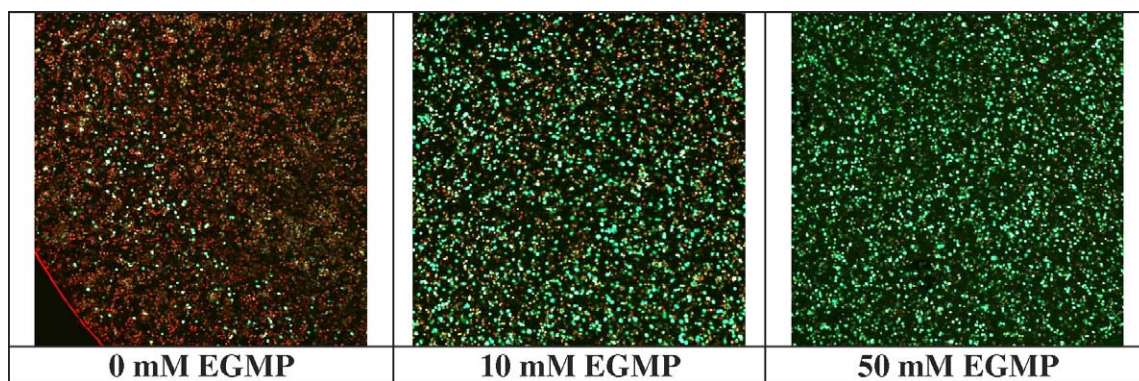


Fig. 8. Effect of charge on hMSC viability inside PEGDA hydrogels. Human MSCs were photoencapsulated in unmodified PEGDA (left, 15% viability), PEGDA w/ 10 mM EGMP (middle, 74% viability), and PEGDA w/ 50 mM EGMP (right, 97% viability). Shown are LIVE/DEAD assay results 1 week after cell encapsulation. Cell viability was improved in the presence of increasing concentrations of EGMP ($\times 50$ original magnification).

3. Experimental procedures

3.1. Human mesenchymal stem cell culture

Human mesenchymal stem cells (hMSCs) were obtained from Cambrex Bio Science (Walkersville, MD) and used as received. Cells were cultured in hMSC growth media [10% fetal bovine serum, 0.50 $\mu\text{g/ml}$ fungizone, 50 U/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, and 20 $\mu\text{g/ml}$ gentamicin in low glucose Dulbecco's Modified Eagle Media (DMEM, Invitrogen) supplemented with 1 ng/ml basic fibroblast growth factor (bFGF, Sigma)]. Cells were split 1:6 approximately once a week and fed every 3 or 4 days until utilized, and cells that had been passaged twice were used for encapsulations.

3.2. Gel preparation

The polymer, or macromer, solution was prepared by dissolving di-acrylated poly(ethylene glycol) (PEGDA) of molecular weight 3400 (Nektar Therapeutics) in phosphate buffered saline solution at a concentration of 100 mg/ml (10 wt.%). The photoinitiating molecule D2959 (Ciba-Geigy) was added to a final concentration of 0.05 wt.%. The solution was then sterilized by filtering through a 0.2- μm syringe filter. Under photopolymerization conditions (i.e., ultraviolet light of intensity 5 mW/cm² for 10 min of exposure), the short-term cytocompatibility (i.e., within 1 day) of this base PEGDA polymer solution has been shown to be suitable for the photoencapsulation of fibroblasts (Bryant et al., 2000), chondrocytes (Bryant and Anseth, 2001), osteoblasts (Burdick and Anseth, 2002; Burdick et al., 2002), and hMSCs (Nuttelman et al., 2004).

3.3. PEG–RGD hydrogels: effect on two-dimensional hMSC attachment

To investigate the effect of incorporating the cell-adhesive RGD peptide sequence (arginine–glycine–aspartic acid) on hMSC attachment to the PEGDA hydrogel, two-dimensional hydrogel disks were made and hMSCs seeded on the surface. A peptide synthesizer (Applied Biosystems, model 433A) was used to synthesize the peptide sequence GRGDS (glycine–arginine–glycine–aspartic acid–serine, N-terminus to C-terminus). This peptide sequence was then covalently linked to a mono-acrylated PEG of molecular weight 3400 containing an *N*-hydroxysuccinimidyl group on one end (Acryl–PEG3400–NHS, Nektar Therapeutics) by reacting equimolar amounts of Acryl–PEG3400–NHS and GRGDS in 0.1 M sodium carbonate buffer at pH 8.5 for 2 h at room temperature. Upon reaction, the product was dialyzed against water overnight using dialysis tubing of molecular weight cutoff 1000 Da. The dialyzed product was then lyophilized to obtain the mono-vinyl PEG with a pendant RGD sequence (Acryl–PEG3400–RGD). Acryl–PEG3400–RGD was dis-

solved in a 10 wt.% PEGDA macromer solution at a concentration of 2.8 mM, and polymerized between two glass slides to form hydrogel “sheets”. Disks (diameter=10 mm, thickness=1 mm) were formed by punching out with a hole punch. The disks were then seeded with hMSCs at a concentration of 5000 cells/cm², and cultured in CON media for up to 48 h to investigate cell attachment. At 4, 24, and 48 h, the two-dimensional surfaces were treated with the LIVE/DEAD assay (Molecular Probes) according to the manufacturer's instructions and analyzed for cell attachment and spreading using fluorescence microscopy.

3.4. Photoencapsulation of hMSCs

When confluent, hMSCs were trypsinized from culture, collected, and centrifuged to pellet the cells. Each cell pellet was then mixed with a 10 wt.% PEGDA solution and adjusted so that the final cell concentration in the hydrogels was 25 million cells/ml. The cell/polymer suspension was mixed carefully to minimize bubble formation, and then 40 μl of the cell/polymer suspension was pipetted into 1-ml sterile syringes that had the tips cut off. The cell/polymer suspensions were then photopolymerized for 10 min under ultraviolet light of intensity ~ 5 mW/cm². Upon polymerization, disks (diameter=5 mm, thickness=2 mm) were pushed out of the syringe using the plunger and placed in hMSC control (CON) media (hMSC growth media without the bFGF) and cultured at 37 °C and 5% CO₂.

3.5. PEG–RGD hydrogels: effect on three-dimensional hMSC culture

Human MSCs were photoencapsulated in a 10 wt.% PEGDA macromer solution containing either 0 or 2.8 mM Acryl–PEG3400–RGD and cultured in hMSC CON media. After 1 week, constructs were removed from culture and 200- μm sections were cut using a vibratome. The LIVE/DEAD assay was then used to visualize the distribution of living and dead cells in interior sections (i.e., midway between the top and bottom) within the hydrogel constructs using laser scanning confocal microscopy (Zeiss Axioplan 2 with LSM 5 Pascal laser), and light micrographs were obtained using an attached camera.

3.6. Mineralization assay

At various time points gels were removed from the DMEM solutions, rinsed three times with PBS, and analyzed for calcium deposition using an assay based on *o*-cresolphthalein complexone and 8-hydroxyquinone. Each sample was incubated in 1 ml 0.6 N HCl overnight at 4 °C. The next day, 2 μl of the supernatant was combined with 8 μl dH₂O and this was added to 100 μl of a solution containing 1 part calcium binding reagent (0.024% *o*-cresolphthalein complexone and 0.25% 8-hydroxyquinone in dH₂O) and 1 part calcium buffer (500 mmol/L 2-amino-2-

methyl-1,3 propanediol in dH₂O). The absorbance of each solution was then measured at 560 nm using a plate reader, and based on a standard curve of known concentrations of calcium chloride, the total amount of calcium deposited in each hydrogel could be determined.

3.7. Osteopontin adsorption to pre-mineralized PEG hydrogels

PEG hydrogels (10 wt.%) were synthesized and mineralized in osteogenic stem cell media [OST, consisted of CON media supplemented with 100 nM dexamethasone (Sigma), 10 mM β -glycerophosphate (Aldrich), and 0.05 mM ascorbic acid phosphate (Sigma)] for up to 10 days. Three samples of increasing mineralization were made by removing hydrogels at different time points. The calcium content of several of the gels of each sample was analyzed using the calcium assay described above. These gels were found to have the following calcium contents: 1) 0 $\mu\text{g Ca}^{2+}$ /mg gel, 2) $11 \pm 2 \mu\text{g Ca}^{2+}$ /mg gel, and 3) $25 \pm 3 \mu\text{g Ca}^{2+}$ /mg gel. In triplicate, the following procedure was performed on each composition of gels: in 1-ml Eppendorf tubes gels were 1) rinsed in phosphate buffered saline (PBS) for 1 h, 2) incubated in 0.1% Tween-20 for 15 min, 3) rinsed in PBS for 1 h, 4) incubated with osteopontin (R and D Systems, 1:100 dilution) for 1 h, 5) transferred to new Eppendorf tubes and blocked in a 3% bovine serum albumin (BSA) solution in PBS for 1 h, 6) incubated with primary antibody (mouse anti-human osteopontin, R and D Systems, 1:1000 dilution) for 1 h, 7) rinsed with 1% BSA in PBS 3 \times for 10 min each, 8) incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, 1:1000 dilution) in 1% BSA/PBS for 1 h, 9) rinsed with PBS for 1 h, 10) transferred to new Eppendorf tubes and incubated in colorimetric solution (R and D Systems) for 30 min, then 11) 0.5 ml 2 N H₂SO₄ was added to stop the reaction. Finally, the absorbance of each solution was measured at 405 nm using a plate reader. The relative adsorption of osteopontin to each of the three gels of varying mineralization could then be determined. Human MSCs were then seeded onto the pre-mineralized PEG hydrogels to evaluate their ability to adhere and attach to gels of varying pre-mineralization.

3.8. Phosphate-containing gels for osteopontin sequestering

Prepolymer solutions containing 10 wt.% PEGDA, 0.05 wt.% photoinitiator I2959 (Ciba-Geigy), and various compositions of ethylene glycol methacrylate phosphate (EGMP, Sigma) were prepared. EGMP compositions were as follows: 0, 10, 100, 1, 5, and 10 mM. Polymer samples of these six prepolymer solutions were made by placing 40 μl of prepolymer solution into 1-ml syringes. Samples were polymerized under ultraviolet light ($\sim 5 \text{ mW/cm}^2$) for 10 min, and disks of each EGMP concentration were made in triplicate for later analysis. Each disk was then placed in 4

ml CON media for 2 weeks at 37 °C to nucleate mineralization and the extent of mineralization was analyzed as described below.

3.9. Human MSC attachment to phosphate-gel surfaces

The photoreactive, charged molecule ethylene glycol methacrylate phosphate (EGMP) was copolymerized into the PEGDA hydrogel network at a concentration of 50 mM. EGMP is negatively charged under physiological pH (7.4). To prepare the copolymer solutions, EGMP was dissolved into a 10 wt.% PEGDA solution in phosphate buffered saline containing 0.05 wt.% photoinitiator D2959 (Ciba-Geigy). Two-dimensional gel disks were made by polymerizing hydrogel “sheets” for 10 min under ultraviolet light (5 mW/cm^2) between two glass slides. Hydrogel disks (diameter=10 mm, thickness=1 mm) were punched out of the sheet using a hole punch, and disks were placed in hMSC CON media for 24 h prior to cell seeding. When confluent, hMSCs were trypsinized from culture and seeded onto hydrogel disks at a concentration of 5000 cells/cm². At 4, 24, and 48 h, seeded two-dimensional surfaces were stained using the LIVE/DEAD assay, and cell attachment, spreading, and viability were analyzed using fluorescence microscopy.

3.10. Human MSC viability in osteopontin-sequestering gels

The effect of incorporating negative charge into the PEGDA network on cell viability of encapsulated hMSCs was investigated by directly polymerizing hMSCs in the presence of a 10 wt.% PEGDA macromer solution containing 0, 10, or 50 mM EGMP. Cell/polymer constructs were cultured for 1 week in CON media. Additionally, several 0 mM EGMP gels were cultured in OST media. After 1 week, cell/polymer constructs were removed from culture, sectioned with a vibratome into 200- μm sections, and sections midway between the top and bottom gel surfaces were stained using the LIVE/DEAD assay. Cell viability and distribution were visualized using a laser scanning confocal microscope.

4. Conclusions

In three dimensional culture (i.e., cells photoencapsulated in PEGDA hydrogels), the viability of encapsulated hMSCs was improved using two different techniques. First, the enhancement of integrin–matrix-like interactions provided directly by tethering the RGD sequence to the network or indirectly by incorporating phosphate groups into the network that facilitate mineralization and subsequent osteopontin adsorption increased overall viability. Second, viability of photoencapsulated hMSCs was dramatically improved by encapsulating cells in the presence of 50 mM EGMP. EGMP is thought to aid in

nucleation of mineral regions, which, under some conditions, can occur within a day of culture. In the absence of EGMP and any heterogeneous mineralized regions within the hydrogel, it is believed that osteopontin produced and secreted by encapsulated hMSCs quickly diffuses away from the cell surface, preventing any sort of cell–matrix interactions. However, if mineralized regions have formed, either by incorporating EGMP or culturing in osteogenic media (OST), which contains soluble mineralization nucleators, it is believed that osteopontin that is secreted from the cell will adsorb immediately on the outside of the cell to the charged mineralized regions. Ultimately, we aim to develop a photoencapsulation system where soluble mineralization nucleators, such as β -glycerophosphate found in OST media, are not needed since it is presumed that these types of small molecule nucleators would not be present at the site of a bone implant. Tethering EGMP initiates mineralization of the network, and as a result of this mineralization, cell viability is improved tremendously.

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